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Title: Concentration gradient of CXCL10 and CXCL11 between the cerebrospinal fluid and plasma in children with enteroviral aseptic meningitis

Authors: Anamarija Čavčić ^a, Goran Tešović ^{b,c*}, Lana Gorenec^c, Ivana Grgić^c, Branka Benić^c, Snježana Židovec Lepej^c

Affiliation:

^aClinical Hospital Center Zagreb, Kišpatićeva 12, 10 000 Zagreb, Croatia

^bUniversity of Zagreb School of Medicine, Šalata 3, 10 000 Zagreb, Croatia

^cUniversity Hospital for Infectious Diseases „Dr. Fran Mihaljević“, Zagreb, Croatia

***Corresponding author.** Goran Tešović, MD, PhD, University Hospital for Infectious Diseases „Dr. Fran Mihaljević“, Mirogojska 8, Zagreb, Croatia, fax: +385 1 2826 158; phone + 385 1 2826 156; e-mail: goran.tesovic@zg.htnet.hr

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Running head: chemokines in enteroviral aseptic meningitis

Abstract

Background: Lymphocyte migration from the blood into the CNS is mediated by chemokines and chemokine receptors. Chemokines CXCL10 and CXCL11 are important for the recruitment of CXCR3-expressing Th1 lymphocytes to the site of inflammation.

Aims: To determine the concentrations of CXCL10 and CXCL11 in the CSF and plasma of children with enteroviral aseptic meningitis (EV AM) and controls and the contribution of these chemokines to the chemokine concentration gradient between the periphery and the CNS.

Methods: The study included 26 pediatric patients with EV AM and 16 controls in whom CNS infection is excluded by negative CSF examination. Chemokines were quantified by using enzyme immunoassay. Etiological diagnosis of EV AM was based on the detection of enteroviral RNA in the CSF using real-time PCR.

Results: CXCL10 (median 12 725 pg/ml) and CXCL11 (median 187 pg/ml) concentrations in CSF of patients with meningitis were significantly higher compared to plasma (median 173 pg/ml and median 110 pg/ml; $p < 0.001$, $p = 0.026$ respectively). CXCL10 concentrations in the CSF (median 198 pg/ml) and plasma of controls (median 124 pg/ml) were not significantly different ($p = 0.642$). CXCL11 concentrations in the CSF of controls (median 89 pg/ml) were significantly lower compared with plasma (median 139 pg/ml, $p = 0.004$). Chemokine concentration gradient was not influenced by pleocytosis, nor dependent on cytologic CSF formula or the presence of proteinorrachia.

Conclusion: CXCL10 and CXCL11 concentration gradient between the CSF and plasma in children with EV AM suggests an important role of these chemokines in the T-cells recruitment into the CNS and local immunoreaction.

Key words: aseptic meningitis, enteroviruses, chemokines, CXCL10, CXCL11

Introduction

Enteroviruses (EV), RNA viruses belonging to *Picornaviridae* family, causes wide spectrum of illnesses, from benign and self-limiting diseases manifested by fever alone to severe life threatening infections, including neonatal sepsis, myocarditis, encephalitis and myelitis.^{1, 2, 3} However, the most common clinical presentation of EV disease that deserves the attention of clinicians especially during the summer and fall season is aseptic meningitis (AM).^{1, 4} The importance of EVs in AM ethiology clearly underlines the fact that more than 80% of all AM cases in which the causative agent was defined are caused by one of those viruses.¹ The widespread use of standardised real-time polymerase chain reaction (PCR) assays for ethiological diagnostics of neurotropic viruses in the cerebrospinal fluid (CSF) of patients with AM has further increased the number of diagnosed cases and thus confirmed the leading position of EVs in the ethiology of this syndrome.¹ Although the clinical characteristics and laboratory findings in EV AM have been well studied, the knowledge of the local immune response is still scarce.^{1, 4 - 6} The majority of studies dealing with this topic have focused on patients with more severe forms of CNS inflammation caused by EVs, especially those with enterovirus 71 (EV71) brain stem encephalitis.^{7, 8}

The leading, although nonspecific laboratory hallmark of EV AM is pleocytosis, usually predominantly lymphocytic.⁴ Some earlier studies demonstrated a preferential recruitment of memory CD4+ T-cells from the blood to the CSF.^{5, 9, 10} These results support the hypothesis that activated memory CSF CD4+ T-cells are the principal cellular subpopulation responsible for the local immune response in patients with inflammatory CNS diseases, including those with EV AM.⁵

After crossing the blood brain barrier (BBB) memory CD4+ T cells can encounter and recognize their specific antigen and trigger the local inflammatory response. Alternatively, in case that memory CD4+ T cells do not encounter an antigen, they will recirculate back into the periphery.¹¹

The lymphocyte migration through the BBB into the CSF in response to infection with neurotropic viruses is mediated by chemokines and their corresponding receptors.¹² Chemokines are small, soluble, basic proteins that can be classified into four subfamilies: CXC, CC, CX3C and C, depending on the number and distribution of cystein residues within the amino terminus of the molecule.¹³ The current hypothesis holds that upon recognition of viral DNA or RNA, Toll-like receptors activate intracellular signaling cascade resulting in enhanced expression of genes coding for various cytokines, particularly interferons and

chemokines.¹⁴ *In vitro* and *in vivo* studies showed that astrocytes and microglia cells are the principal sources of chemokines following viral infections of the CNS. Studies in both animal and human models showed complex and variable patterns of chemokine synthesis in various CNS viral infections.¹⁴

Chemokines CXCL10 (interferon-inducible protein-10, IP-10) and CXCL11 (interferon-inducible T-cell alpha chemoattractant) are CXC chemokines that are synthesised upon stimulation with IFN- γ during infection or inflammation.¹¹ Both chemokines bind to the chemokine receptor CXCR3 and are important in pathologies mediated by activated Th1 CD4⁺ T-cells. The biological role of CXCL10 appears to be complex. CXCL10 recruits antigen-specific activated CXCR3-positive T-cells into the CNS enabling the efficient control of viral replication.¹³ However, persistent overexpression of CXCL10 can lead to continuous infiltration of inflammatory cells that can subsequently lead to neurotoxicity, cell death or an immune-mediated demyelinating disease.¹⁴⁻¹⁶

Several studies investigated the expression of CXCL10 and CXCL11 as well as possible contribution to the immunopathogenesis of CNS infections caused by tick-borne encephalitis virus, West Nile virus, lymphocytic choriomeningitis virus, human immunodeficiency type 1 virus, herpes simplex virus and neuroborreliosis in adult patients.¹⁷⁻²³ However, literature data on the expression of these chemokines in pediatric viral CNS infections are scarce. The Taiwanese investigators demonstrated the presence of CXCL10 in the CSF of children with brainstem encephalitis developing as a consequence of infection with enterovirus 71.⁸ To our knowledge, the expression of CXCL10 and CXCL11 in the CSF of Caucasian children with non-complicated clinical course of EV AM has not been previously investigated.

The aim of this study was to investigate the presence of CXCL10 and CXCL11 in the CSF and plasma of children with EV AM and compare the observed pattern of expression with controls in whom the diagnosis of CNS inflammatory disease was excluded. The results of this study are expected to determine whether overexpression of CXCL10 and/or CXCL11 in the CSF contributes to the induction of local cellular immune response in patients with EV AM.

Additionally, extensive research on the role of chemokines in the pathogenesis of various inflammatory diseases consequently lead to the development of a large number of molecules targeting chemokines and chemokine receptors as candidates for human therapeutics. So far, two drugs targeting chemokines and chemokine receptors are currently in use; a CCR5 inhibitor for the treatment of HIV-infection and a CXCR4 antagonist that stimulated hematopoietic stem cell mobilisation that is essential for the treatment of hematological

malignancies.²⁴ Therefore, the results of this study can provide an important insight into possible relevance of chemokine/chemokine receptor inhibitors/antagonists as potential therapeutics in the treatment of inflammatory CNS diseases.

Patients and methods

Study design and patients:

This prospective, cross-sectional study was carried out at the University Hospital for Infectious Diseases „Dr. Fran Mihaljevic“ (UHID), Zagreb, Croatia between January 2009 and November 2010. The study included 42 patients aged less than 14 years of whom 26 with EV AM, and 16 controls. The diagnosis of AM was based on clinical signs and symptoms, accompanied with CSF cytological and biochemical findings, negative Gram stain and bacteriological culture.²⁵ The EV etiology was confirmed using real-time PCR. The control group of patients consisted of children with clinical suspicion of CNS infection at the admission to the hospital, but in whom the initial diagnosis was ruled out based on negative CSF cytology, bacteriological analysis, as well as the exclusion of EV infection using panenteroviral real-time PCR. In all patients included in the control group an extraneural site of infection was subsequently proved. The final diagnosis were as follows: streptococcal pharyngitis, pneumonia and viral gastroenteritis - four patients per diagnosis, while influenza A and urinary tract infection were diagnosed in three and one patient, respectively.

The Ethics Committee of the UHID approved this study and informed consent was obtained from all parents or custodians.

Samples:

Chemokines CXCL10 and CXCL11 were quantified in CSF and plasma samples collected on the same day in all patients and controls. All samples for analysis were taken within 24 hours after the admission. CSF samples (volume between 400-1000 µL) were collected by lumbar puncture (LP) and stored at -80°C until testing in two aliquots to avoid repeated freeze and thaw cycles. Plasma obtained from peripheral blood samples collected with K₃EDTA was also stored at -80°C until analysis.

The number of cells per µl of CSF was determined by counting in a Fuchs-Rosenthal chamber. CSF sediments were prepared by using a Shandon Cytospin 3 (Life Sciences International Europe, Astmoor, Runcorn, Cheshire, UK) for 5 min. at 300g. One CSF sediment sample was stained with My-Grunwald-Giemsa stain for routine cytological analysis. Percentages of granulocytes, lymphocytes and monocytes were determined after analyzing 200 cells in the sediment. Biochemical analysis was performed by using Beckman Coulter AU640 (Beckman Coulter, Fullerton, CA, USA).

Chemokine quantification

Concentrations of CXCL10 and CXCL11 in the CSF and plasma of patients as well as controls were determined by using standardised enzyme immunoassays Quantikine Human CXCL10/IP-10 ELISA and Quantikine Human CXCL11 ELISA (R&D Systems, Minneapolis, USA).

Etiological diagnostics

Detection of pan-enteroviral RNA in the CSF of all patients and controls was performed by using real-time PCR. RNA was extracted from the 200 µL of CSF by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The presence of EV RNA was determined by using Cepheid Analyte Specific Reagent (ASR) EV assay on real-time PCR SmartCycler instrument (Cepheid, Sunnyvale, CA, USA). Cepheid ASR EV assay contains FAM-labeled and Alexa 532-labeled probe that are designed to detect a 115 bp sequence of the highly conserved 5'untranslated region (UTR) of EVs.

Statistical analysis

Kolmogorov-Smirnov test was used to test the normality of quantitative data. Comparison between the two independent groups was made with nonparametric Mann-Whitney U test while the Wilcoxon signed ranks test was used for paired data. Fisher exact test was used for categorical data and correlation between quantitative data was determined using Pearson correlation coefficient. Statistical significance was determined at the 0.05 level. Statistical analysis was performed by using SPSS (Statistical Package for Social Sciences) version 13.5 (SPSS Inc., Chicago, IL, USA).

Results

There were 42 patients included in the study, of whom 26 (61.9%) with confirmed EV AM and 16 (38.1%) in the control group. In EV AM group there were 17 (65,4%) males, while in the control group males represented 62,5% of all patients. The median age of patients in the EV AM group was 72.0 months (range: 1-168 months) whereas in the control group the median age of patients was 31.5 months (range: 3-132 months). Comparing the main characteristics (sex, age) between EV AM and control group, no statistical difference was found ($p>0.999$ and $p=0.140$, respectively) (Table 1.). The differences were also not found comparing the day of illness when LP was done, maximum fever, as well as peripheral white blood cell (WBC) count, while there were statistically significant differences in comparison of C-reactive protein (CRP) concentrations in plasma, the amount of pleocytosis as well as CSF protein concentrations between the two groups (Table1.)

Concentrations of CXCL10 in the CSF of patients with EV AM (median 12 725 pg/ml, range 235-174 439 pg/ml) were significantly higher compared to those from the corresponding plasma samples (median 173 pg/ml, range 8-5 192 pg/ml) ($p<0.001$). CXCL10 concentrations in the CSF (median 198 pg/ml, range 21-436 pg/ml) and plasma (median 124 pg/l, range 43-686 pg/ml) of patients from the control group were not significantly different ($p=0.642$).

Similarly, concentrations of CXCL11 in the CSF of patients with EV AM (median 187 pg/ml, range 0-6272 pg/ml) were significantly higher in comparison to plasma concentrations (median 110 pg/ml, range 52-937 pg/ml) ($p=0.026$). Contrary to CXCL10 pattern of expression, CXCL11 concentrations in the serum of patients from the control group (median 139 pg/ml, range 71-1969 pg/ml) were higher compared to their corresponding CSF concentrations (median 89 pg/ml, range 0-274 pg/ml) ($p=0.004$). Data on CXCL10 and CXCL11 concentrations in patients with EV AM and controls are shown in Figure 1.

Analysing the possible contribution of amount of pleocytosis on chemokine concentrations in the CSF, the positive correlation were not found for CXCL10 and CXCL11 in patients with EVAM ($r=-0.142$, $p=0.488$ and $r=-0,100$, $p=0.961$, respectively) nor in patients from the control group ($r=0,444$, $p=0.085$ and $r=0.134$, $p=0.621$, respectively). The comparison of CXCL10 and CXCL11 concentrations in patients with EV AM in relation to cytologic CSF formula (polynuclear vs lymphocytic predominance), as well as in relation to

absence/presence of proteinorrachia (cut off value 450 mg/L) was also done. We found no difference in CXCL10 and CXCL11 concentrations among those groups of patients (Table 2.).

Discussion

The results of this study showed, for the first time, that CSF of children with EV AM contains chemokines CXCL10 and CXCL11. This study demonstrated increased concentrations of CXCL10 and CXCL11 in the CSF of patients with EV AM compared with plasma as well as the absence of such concentration gradient in patients from the control group.

CXCL10 is a CXCR3-ligand that enables recruitment of activated T-cells to sites of immune reactions, mostly inflammation. The prerequisite for the migration of CXCR3-positive cells to sites of inflammation is the concentration gradient of CXCL10. Several studies conducted on animal models as well as in adult patients with neurotropic viral infections showed increased expression of CXCL10 in the CNS/CSF compared with peripheral blood.¹⁷⁻²¹

To our knowledge, the only study on chemokine expression in the CSF of pediatric patients with enteroviral CNS infection was published in 2008 by Wang et al.⁸ The study conducted in Taiwan focused on expression of CXCL10, CXCL9, CCL5, CCL2 and interleukin-8 in the CSF and plasma of children with brainstem encephalitis (BE), a serious neurological manifestation of EV71 infection.⁸ Increased levels of CXCL10, CXCL9, CCL2 and IL-8 in the plasma of children with pulmonary edema (PE) versus uncomplicated BE demonstrated an association between the extent of chemokine response and clinical presentation of disease.⁸ The results of our study can not be directly compared with Wang's mainly due to the fact that our study enrolled children with uncomplicated AM associated with various EVs (not limited to EV71). However, contrary to our results, a concentration gradient between CSF and plasma concentrations of CXCL10 was not observed in children with BE caused by EV71 irrespective of clinical presentation (uncomplicated BE, autonomic nervous system dysregulation and PE). The observed difference confirms previous findings on distinct chemokine signature patterns observed in CNS viral infections.¹⁴

The presence of CXCL10 concentration gradient between the CSF and plasma of children with EV AM supports the concept that CXCL10 might act as a chemoattractant for antigen-specific activated T-cells and play an important role in their recruitment into the CSF. This hypothesis can be indirectly confirmed by observing a different pattern of CXCL10 expression (no difference in CSF/plasma concentrations) in controls, e.g. children in whom the initial clinical diagnosis of CNS inflammation was subsequently excluded. To our knowledge, our study is the first to provide experimental evidence on CXCL10 and CXCL11

quantification in the CSF of pediatric controls (only plasma concentrations were reported in other studies).¹⁴

Literature data on CXCL11 expression in the CSF of patients with inflammatory and non-inflammatory CNS diseases are limited to TBE, neuroborreliosis and multiple sclerosis (MS)^{17, 22, 23, 26, 27} Rupprecht et al. showed significantly higher expression of CXCL10 in the CSF of patients with neuroborreliosis compared with controls as well as a correlation between CXCL11 levels and CSF-white cell counts.²³ More recently, Szczucinski et al. found no difference in the levels of CXCL11 in the CSF of patients with active or stable relapsing-remitting multiple sclerosis (MS) compared with controls.²⁶ However, in Mellergard's study a significant decline in CSF CXCL10 concentrations in MS patients after one year of natalizumab treatment was observed.²⁷ Our study provides the first experimental evidence on the CXCL11 concentration gradient between the CSF and plasma in patients with EV AM. The severity of local inflammation and degree of BBB dysfunction which were in our patients measured only indirectly through the analysis of total CSF cell count, CSF polynuclear predominance and the presence/absence of proteinorachia probably has no impact on chemokine gradient existence. However, further studies are needed prior to final conclusion could be done.

Additionally, studies on the expression of selected chemokines in patients with non-complicated clinical course of EV AM versus patients with adverse clinical course might help establish a rationale for anti-chemokine therapy in severe enteroviral CNS infections, especially encephalitic forms of disease and possibly other inflammatory CNS diseases in the future.

In conclusion, this study demonstrated the presence of CXCL10 and CXCL11 concentration gradient between the CSF and plasma in children with uncomplicated clinical course of EV AM suggesting an important role of these chemokines in the recruitment of T-cells into the CNS and local immune response to EV.

Further studies on a greater number of patients including those with encephalitic form of disease are needed. In case similar findings are reached, it could be a further support to the hypothesis that chemokines/chemokines receptors may serve as therapeutical targets in severe forms of viral CNS diseases as well as possible surrogate markers in predicting the severity of disease.

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Table 1 - Main clinical and laboratory characteristics of patients with enteroviral aseptic meningitis (EV AM) and controls

Parameter	EV AM	Controls	p-value
Sex	17 M; 9 F	10 M; 6 F	>0.999
Age in months; median (range)	72.0 (1 – 168)	31.5 (3 – 132)	0.140
Day of illness when LP was performed; median (range)	2.0 (1.0 – 4.0)	2.0 (1.0 – 5.0)	0.718
Maximum fever in °C; median (range)	38.3 (37.6 – 40.0)	38.9 (37.0 – 39.8)	0.231
CRP in mg/L; median (range)	4.35 (0.2 – 23.8)	10.7 (0.2 – 144.9)	0.029
WBC x 10 ⁹ /L; median (range)	12.1 (5.5 – 27.8)	12.5 (2.5 – 56.5)	0.400
CSF pleocytosis cells/μL; median (range)	130.5 (90.0 – 2386.0)	3.0 (1.0 – 6.0)	<0.001
CSF proteins mg/L; median (range)	528 (230 – 1034)	236 (185 – 615)	<0.001

- LP, lumbar puncture; WBC, white blood cells; CSF, cerebrospinal fluid

Table 2 - CXCL10 and CXCL11 concentrations in different subgroups of patients with enteroviral aseptic meningitis (EV AM)

Chemokine	Cerebrospinal fluid formula		p-value	CSF protein concentration		p-value
	PML<50%	PML≥50%		<450 mg/L	≥450 mg/L	
CXCL10 pg/mL; median (range)	13878 (235–75439)	10930 (3189–102090)	0.470	15402 (1637– 27823)	11251 (235– 175439)	0.712
CXCL11 pg/mL; median (range)	187 (0-6282)	188 (99 – 6126)	0.795	153 (70– 1613)	211 (0 – 6272)	0.617

- PML, polymorphonuclear leukocytes

Figure 1. Concentrations of CXCL10 and CXCL11 in the cerebrospinal fluid of children with enteroviral aseptic meningitis and controls

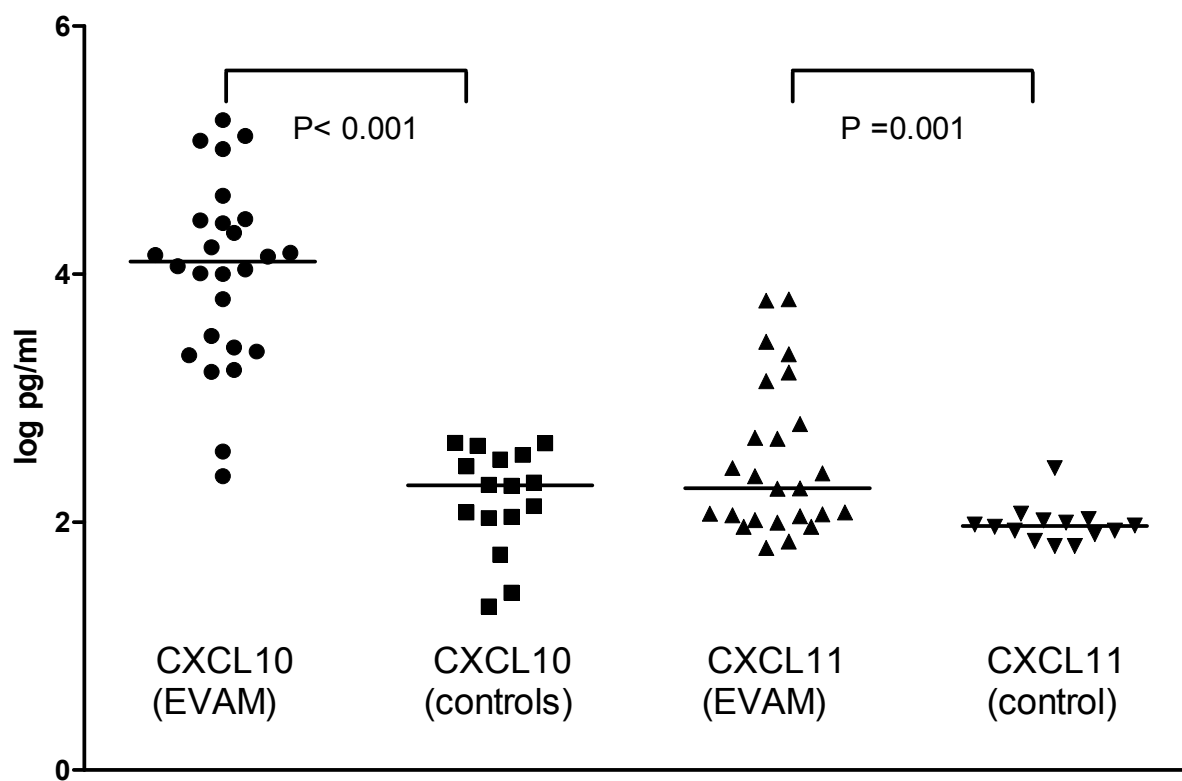


Figure 1. Concentrations of CXCL10 (log₁₀ pg/ml) and CXCL11 (log₁₀ pg/ml) in the cerebrospinal fluid of children with enteroviral aseptic meningitis (EVAM) and controls. In two patients in the control group and one in the EVAM group, CXCL11 was not detected and these measurements are excluded from the graph. The horizontal line is the median value.